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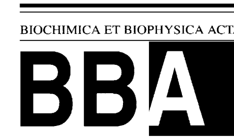

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Mechanistic aspects of CPP-mediated intracellular drug delivery: Relevance of CPP self-assembly

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Abstract

In recent years, cell-penetrating peptides have proven to be an efficient intracellular delivery system. The mechanism for CPP internalisation, which first involves interaction with the extracellular matrix, is followed in most cases by endocytosis and finally, depending on the type of endocytosis, an intracellular fate is reached. Delivery of cargo attached to a CPP requires endosomal release, for which different methods have recently been proposed. Positively charged amino acids, hydrophobicity and/or amphipathicity are common to CPPs. Moreover, some CPPs can self-assemble. Herein is discussed the role of self assembly in the cellular uptake of CPPs. Sweet Arrow Peptide (SAP) CPP has been shown to aggregate by CD and TEM (freeze-fixation/freeze-drying), although the internalised species have yet to be identified as either the monomer or an aggregate.
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1. Introduction

Recent advances in genomics and proteomics have led to numerous interesting new drug candidates of various types, such as short oligonucleotides, plasmids, peptides or proteins. Unfortunately, these compounds are unable to cross the cell membrane, unless specific mechanisms have evolved with this objective. Thus, carrier delivery methods have to be developed to impart good bioavailability to these compounds. Different approaches have been described to deliver drugs into cells (e.g., micro-injection, electroporation, freeze–thaw techniques, viral delivery systems, liposomes or cationic lipids) but these methods suffer from problems such as low transfer efficiency, complex manipulation, cellular toxicity or immunogenicity.

An alternative delivery method is the use of peptides able to translocate the cell-membrane, these peptides are often referred as cell-penetrating peptides (CPPs). Work in this area stemmed from the discovery that the third helix of Antennapedia homeodomain, pAntp(43–58), can cross biological membranes [1]. Since then, different CPPs have been described to efficiently deliver various types of cargo to the inside of cells, from low molecular weight drugs to liposomes, plasmids, antibodies or nanoparticles [2].

Initially, CPPs were defined as short cationic peptides able to translocate through the plasma membrane of eukaryotic cells via a receptor- and endocytosis-independent mechanism, but a re-evaluation of the internalisation mechanism yielded a new CPP concept. CPPs are peptides made of less than 30 amino acids that are internalised via energy-dependent or independent mechanisms. Positively charged amino acids, hydrophobicity

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Table 1
Principal classes of CPPs with their characteristics

Name	Sequence	Length	Net charge	Kind of amphipathicity	Aggregation?	Internalisation mechanism
Natural CPPs						
Tat(48–60) [a]	GRKKRRQRRRPPQ	13	8	Secondary amphipathicity		Endocytic
Penetratin (pAntp(43–58))	RQIKIWFQNRRMKWKK	16	7	Partially secondary amphipathicity	Yes	Endocytic
MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	27	5	Primary amphipathicity		Independent of endocytosis
SAP (Sweet Arrow Peptide)	VRLPPPVRLPPPVRLPPP	18	3	Secondary amphipathicity	Yes	Endocytic
hCT(9–32)-br	{[LGTYTQDFNK(&)FHTFPQTAIGVGAP] [PKKKRKVEDPGVGFA&]} [b]	40	5			Endocytic
Artificial CPPs						
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	27	5		Yes	Endocytic
MAP (Model Amphipathic Peptide)	KLALKLALKALKAALKLA	18	5	Secondary amphipathicity	Yes	Independent of endocytosis
pVEC	LLIILRRRIRKQAHAHSK	18	8			Independent of endocytosis
Pep-1	KETWWETWWTEWSQPKKKRKV	21	6	Primary amphipathicity	Yes	Independent of endocytosis
Polyarginines	R_n	*n*	n			Unclear

[a] Amino acid sequence could vary with different studies.
[b] See reference [120] for the nomenclature system.

and amphipathicity are common features shared among many of the known CPPs (Table 1). CPPs also include peptides with non-natural amino acids, such as β-amino acids [3] or γ-amino acids [4,5], which have the advantage of being completely stable to proteolytic degradation. Amino-acid based dendrimers capable of crossing the cellular membrane [6] have also been reported.

The development of efficient cell-penetrating peptide vectors demands a precise understanding of the different steps of the internalisation mechanism, the peptide structural features required for every step and the type of cargo to be delivered.

2. Internalisation mechanism

2.1. First step: interaction with the extracellular matrix

In the majority of cases, internalisation begins with interactions between the CPP and the extracellular matrix, requiring the capture of the CPP by cell-surface proteoglycans (PGs). Proteoglycans, the plasma membrane carriers for the majority of CPPs, are a heterogeneous group of proteins substituted with long, linear, polysulphated and negatively charged glycosaminoglycan (GAG) polysaccharides. Chondroitin sulphate (CS), dermatan sulphate (DS) or heparan sulphate (HS) are the most prevalent GAGs in PGs. These proteins feature a common linkage region, GlcA–Gal–Gal–Xyl, where a glycosidic bond is formed between the Xyl residue and a Ser chain from the core protein. The GAG chain may be extended by disaccharides consisting of either GlcA-β-GalNAc or GlcA-α-GlcNAc. The anionic charge of the chains is the result of sulphation reactions [7].

Cell-surface heparan sulphate proteoglycans (HSPGs) are divided into two families: the syndecans and the glypicans. Syndicans are transmembrane HSPGs that are substituted with either chondroitin sulphate or by heparan sulphate, and that position the HS chain distal to the plasma membrane. On the other hand, glypicans are linked through the plasma membrane lipid via a glycosylphosphatidylinositol (GPI) anchor, wherein the HS chains, which only contain HS, are positioned adjacent to the plasma membrane [8,9].

Whether the CPP internalisation occurs via passive diffusion or by endocytosis, it seems that the initial contact between most CPPs and the cell involves the proteoglycans. These physiological membrane constituents act as acceptors, or receptors, for extracellular CPPs, ultimately leading to peptide uptake at multiple sites of the cell surface (Fig. 1(1)).

Different methods have been used to study the role of HSPGs in CPP uptake, including isothermal titration calorimetry [10,11], ESR spectroscopy [12] and affinity chromatography [13]. Furthermore, three kinds of in vitro studies have been used: enzymatic degradation of extracellular HS chains, competition with exogenous HS chains, and HSPG-deficient mutant cells [7,14,15].

The first notion that cell-surface proteoglycans are implicated in the onset of Tat(48–60) internalisation arose from studies done with the full-length Tat protein [14,16–18]. A study by Silhol et al. [19] established that the internalisation mechanism of full-length Tat protein involved HSPGs, even if it was fused to GST or GFP, but this dependence was not found in the case of Tat(48–60) CPP. On the contrary, the majority of recent studies argue for the involvement of HSPGs in the uptake of Tat(48–60). Suzuki et al. [20] demonstrated that incubation of Tat(48–60) with HS or CS-A, CS-B or CS-C – where A, B or C

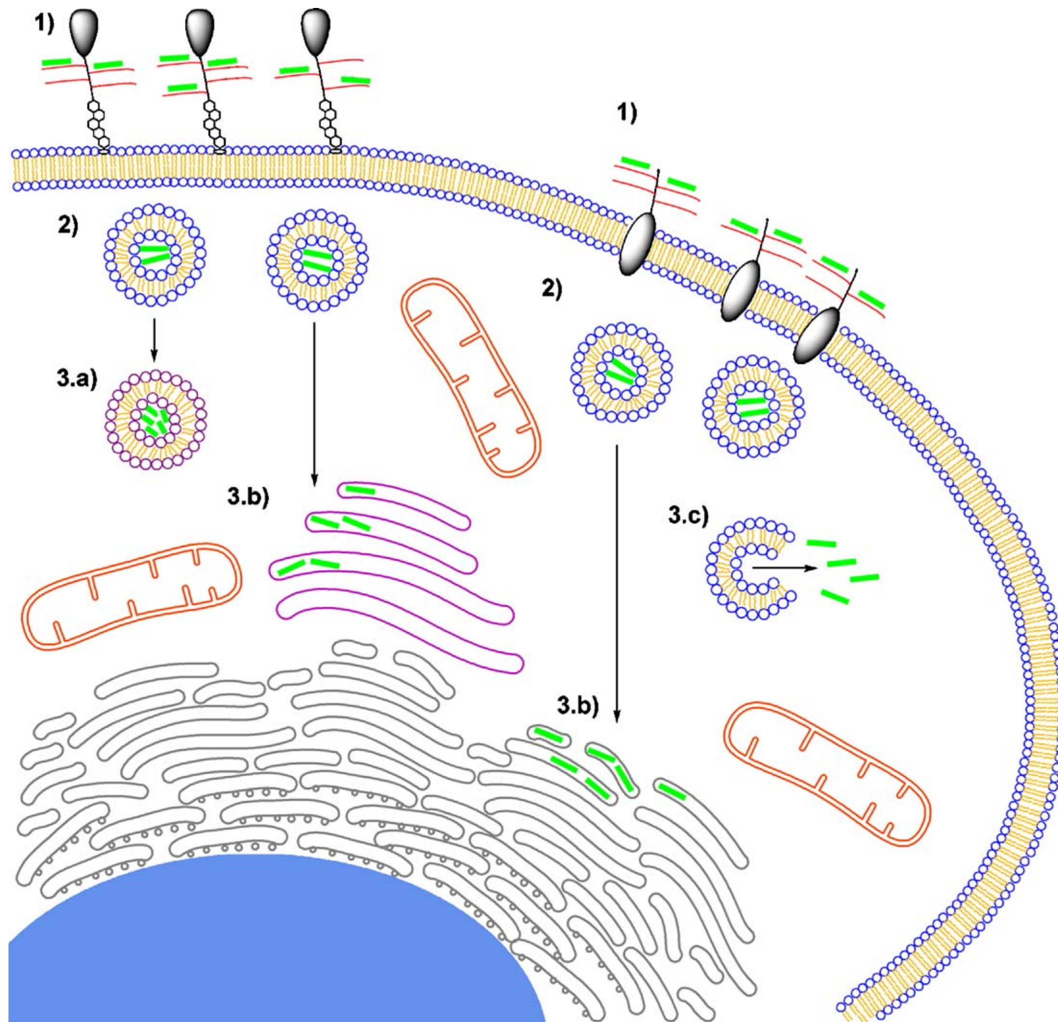

Fig. 1. Different steps in CPP-mediated intracellular delivery. (1) Interaction of the CPP (represented as a green bar) with the cell-surface proteoglycans (in red). (2) Endocytic pathway. (3a) Degradative route to lysosomes in clathrin-mediated endocytosis. (3b) CPP ultimately reach the Golgi apparatus (in purple) or endoplasmic reticulum (ER, in grey) in caveolin-mediated endocytosis. (3c) Endosomal release.

correspond to different positions of the sulphate group – impaired its cellular uptake. The same was found for Arg_8, leading to the conclusion that the electrostatic interaction between the sulphated polysaccharides and the Arg-rich peptides plays a crucial role in cell membrane penetration.

Working with biotinylated CPP complexed with streptavidin or avidin, it was found that coincubation with heparin inhibited internalisation by 60–70% in the case of Tat(46–56) and 40–50% for Antp(43–58) [21]. On the other hand, dextran sulphate inhibited the uptake of Tat(46–56) complex but not that of Antp(43–58) complexes, while CS-A, CS-B, CS-C and hyaluronic acid had no effect on the uptake of Tat (46–56) or Antp(43–58) complexes. Moreover, the complexes were not internalised into mutant CHO cells deficient in GAGs.

The internalisation of Arg_9 labelled with TAMRA fluorescent marker was completely impaired for the case of two mutant CHO cell lines: CHO-pgsD-677, which do not produce HS, and CHO-pgsA-745, which produce neither HS nor CS [13]. The same effect was seen when adding exogenous heparin to the culture medium. Calorimetric studies also support a strong affinity of Arg_9 for HS, with binding stoichiometries close to charge neutrality.

For penetratin (pAntp), the internalisation was favoured in cells expressing α-2,8-polysialic acid (PSA) at their surfaces, although the internalisation was not blocked in cells that do not express PSA [22,23].

Apart from Tat, pAntp and polyarginines, there are other CPPs in which the initial cell-surface interaction is via HSPGs. Recently, it has been shown that the internalisation levels of hCT(9–32)-br and SAP (Sweet Arrow Peptide) are decreased if heparin is added to the cell culture medium [24]. As expected, the decrease is directly proportional to the number of positive charges.

The primary interaction between the Arg-rich CPPs and HSPGs is electrostatic, but it is also likely that hydrogen bonding occurs, taking into account the ability of the guanidinium group to form hydrogen bonds with sulphate and carboxylate groups [25–28] (Fig. 2).

Interestingly, a common peptidic motif has been found for interactions with HSPGs, consisting of basic amino acids flanked by hydrophobic residues. The sequences BBXB and

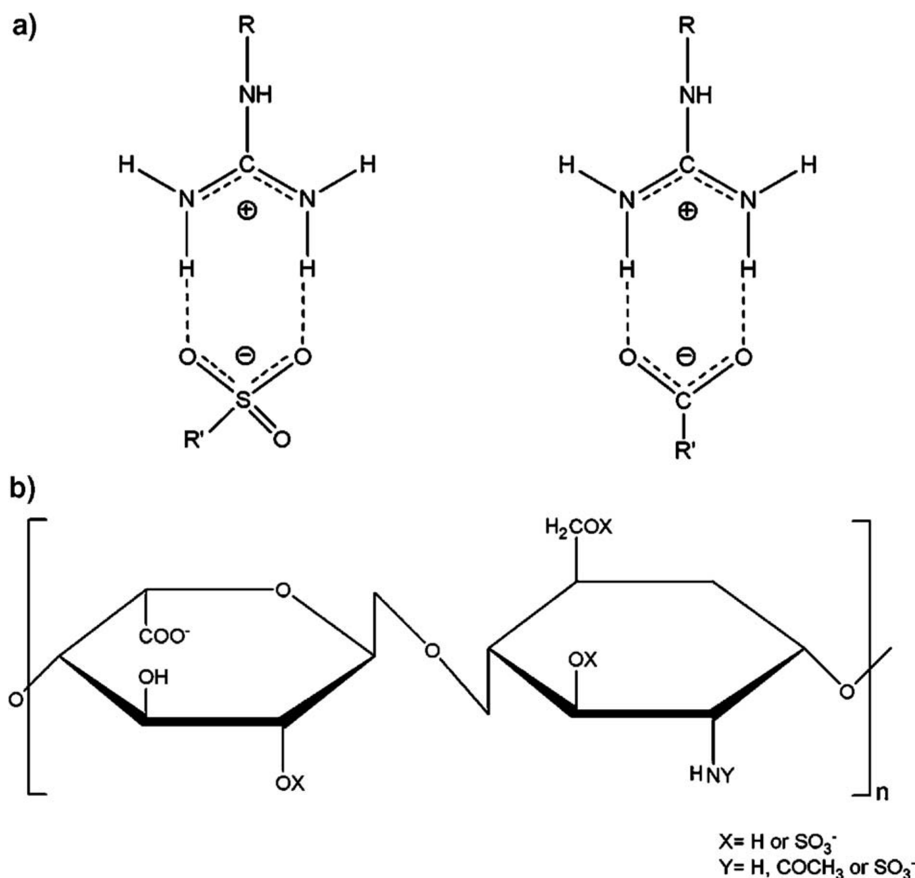

Fig. 2. (a) Interaction of the guanidinium group with sulphates and carboxylates. (b) Structure of heparan sulphate.

BBBXXB – where B = basic amino acid, and X = hydropathic amino acid – have been reported [29,30].

Moreover, it has been described that the binding of a ligand to HSPGs induces aggregation and ligand clustering, such that CPPs are concentrated at the cell surface for subsequent internalisation [11,31]. Recently, these aggregates have been visualised using differential interference contrast CLSM for Tat (46–56) incubated with mouse fibroblasts [31]. If HS is added afterwards, dark aggregates corresponding to the self-assembled peptide disappear from the cell surface appearing in the cell culture medium. When HS is added at the same time as Tat(46–56) CPP, aggregates are not formed at the cell surface, thereby preventing the uptake of Tat(46–56).

Cell-surface HSPGs are known to enter the cell by endocytosis and to be degraded in lysosomes as a major route of turnover [32]. Interestingly, GPI-anchored proteins of glypicans are associated with sphingolipid and cholesterol-rich plasma membrane domains, such as those involved in caveolin-mediated endocytosis [8].

Consistently, after the cell-surface HSPGs have captured the CPP and promoted its concentration at the cell surface, three different mechanisms can follow: direct membrane penetration (the CPP–HSPG complex is enzymatically cleaved and passes directly through the plasma bilayer to the cytoplasm), classical endocytosis or caveolar endocytosis [8].

The binding of CPPs to the cell-surface through negatively charged sulphates or carboxylates has sparked the idea that CPPs internalisation is simply a matter of cell-membrane recycling. More precisely, the CPP attaches to the cell surface and exploits inherent cell-membrane turnover to be internalised [33]. This would be similar to CPP internalisation kinetics, as the cell plasma membrane is described to be renewed very frequently. Concretely, ca. 2–5% of a cell-membrane is recycled every minute [34,35].

The CPP–HSPG interaction is not permanent. Fuchs et al. [13] argued that once the CPP is trapped inside an endosome, the affinity of the interaction between the CPP and HSPGs diminishes because cleavage of HS from the core protein by heparanases causes a decrease in its anionic charge. The resultant released peptide is thus “free” inside the endosome.

The interaction between HSPGs and CPPs was recently exploited to create Vectocell® peptides, a new family of CPPs derived from known heparin binding proteins [36]. Among the different peptides generated, DPV3 has shown the greatest internalisation level, with an even greater value than that for Tat CPP.

Interestingly, another type of drug carrier, cationic lipid reagents (e.g., Lipofectamine or Transfectan), also require cell-surface PGs for optimal transfection efficiency [37–39].

2.2. Second step: translocation through the cell membrane

It was initially thought that CPPs were internalised via a rapid, receptor- and endocytosis-independent mechanism,

namely passive diffusion or inverted micelle formation [1,20,40–44]. However, it was ultimately discovered that the fluorescence diffuse pattern observed by CLSM was an artefact of the cell fixation protocol, which caused an artificial redistribution of the CPP [45]. Moreover, when quantifying CPP uptake by flow cytometry, another kind of artefact was incurred: as flow cytometry does not discriminate between membrane-adsorbed or internalised peptide, it was found that a step to remove the peptide attached to the membrane is needed to avoid overestimation of internalisation [45]. Working with unfixed cells, it was observed that Tat(48–60) and Arg_9 colocalised with transferrin receptor and the endocytic marker FM 4–64, leading to the conclusion that endocytosis could play a role in internalisation. Furthermore, the uptake of both peptides was blocked when the temperature was decreased to 4 °C, or the intracellular level of ATP was depleted using sodium azide and 2-deoxy-D-glucose [45].

Since then, numerous studies have been undertaken to re-examine the internalisation mechanism of CPPs, using live cells instead of fixed cells. In the majority of these experiments, endocytosis has been identified as the most probable pathway for the cellular uptake of CPP (Fig. 1(2)) [13,21,46–50].

The fact that polyarginines, Tat(44–57) or Antp(43–58), are not able to penetrate through lipid bilayers or liposomal membranes may be proof against passive diffusion and in favour of the endocytic mechanism [11,46,51,52]. However, studies claiming the direct passage of polyarginines through the cytosolic membrane in the membrane potential direction have also been reported [53].

In observing in vivo cells by CLSM, two different patterns are found for Tat CPP, Antp and polyarginines: a diffuse and a punctate pattern [49]. Some authors maintain that the observed diffuse fluorescence comes from endocytic release to the cytoplasm and nucleus, but the majority claim that the two patterns found are due to different internalisation mechanisms.

Potocky et al. [54], observing by confocal microscopy the pattern of internalisation of Tat(47–57), found three different internalisation patterns: those with punctuate fluorescence, others with a combination of punctate and diffuse fluorescence, and still others with only diffuse fluorescence, located in the cytoplasm and the nucleus. Strikingly, the fluorescence distribution was concentration-dependent, being mainly punctate at low concentrations of Tat(47–57) and having its highest ratio of diffuse fluorescence at higher concentrations. Again, Tat (47–57) colocalised with transferrin, and was not taken up in the presence of sodium azide and 2-deoxy-D-glucose. Thus, it was believed that the uptake was mainly endocytic. The authors argued that the diffuse fluorescence observed was a consequence of endosomal escape. To prove this hypothesis, they coincubated the peptides with ammonium chloride, a weak base that increases the pH of acidic organelles, including endosomes. After coincubation, Tat(47–57) was only found in endosomes. It was therefore concluded that changes in pH can alter the conformation of the peptide or the formation of its aggregates, thereby enabling it to cross the endosomal membrane.

Re-evaluation of the uptake mechanism for polyarginines was demonstrated with octaarginine. It was shown that the internalisation in fixed cells had a diffuse pattern in the cytoplasm and nucleus, whereas without the fixation protocol the peptide was found in cytoplasmic vesicular structures. Moreover, the uptake was significantly reduced at 4 °C, as judged by flow cytometry analysis [55].

To distinguish between cytoplasmatic or vesicular internalised CPP, two approaches have been used. The first, by Zaro and Shen [56], uses a novel subcellular fractioning method to separate vesicular versus cytoplasmatic compartments, allowing differentiation between endocyted or directly translocated CPP. In CHO cells, $YG(1\text{-}R)_9$ and Tat CPP were mainly translocated (18% and 8%, respectively) while $YG(1\text{-}K)_9$ was mostly endocyted. The authors concluded that membrane translocation requires the guanidinium group of arginine, whereas endocytosis only requires positive charges [56].

Another method for distinguishing between diffuse and punctate internalised CPP is the use of an image analysis algorithm that can quantify fluorescence that originates from cellular vesicles [57]. The granularity algorithm has been used to calculate the mean fluorescence per granule as well as the number of fluorescent granules per cell. The resultant distribution pattern varied with the cell line used and the temperature. For pAntp, the pattern was mostly vesicular for the two cell lines used in the study, however, at 4 °C, there was no visible amount of punctate staining. It was thus concluded that for pAntp, the uptake was mainly endocytic. For R_7 and R_7W, a mixture of diffuse and punctate pattern was observed at 37 °C, whereas at 4 °C no punctate staining was observed. However, once peptidic cargo was attached to the CPP, the uptake pattern was almost completely punctate.

This later aspect is frequently observed; the attachment of large macromolecules often alters the internalisation mechanism. The kinetics of cellular uptake have been shown to be dependent on the size of the cargo attached to the CPP [19,36]: when a high molecular weight cargo is attached, the uptake occurs via endocytosis.

Great effort has been made to characterise the interaction between CPPs and lipids, the main components of the cell-membrane. It was initially thought that the first contact with the cell-surface was due to an electrostatic interaction between the positively charged residues of the CPP and the negatively charged phospholipid head groups. But, in addition to results from in vitro experiments, calorimetric studies demonstrate that, at least for Arg_9 and Tat CPP, the affinity for HS is greater than that for anionic lipid vesicles [10,11,52]. As the first contact is primarily with cell-surface proteoglycans, two options arise for CPP–lipid interaction: if the peptide can insert itself into lipid bilayers, the lipid–CPP bond could be useful for more tightly attaching the CPP to the cell-surface, whereas if the peptide can penetrate a lipid bilayer, endosomal release could be achieved [11,13].

The lipidic composition of the cell-membrane seems to be an important factor in the translocation process; CPP is taken up into mammalian cells far more efficiently than into plant protoplasts [58]. The internalisation of transportan, penetratin or pVEC into Bowes human melanoma cells was significantly greater than that into *N. tabacum* cv. SR-1 protoplasts. The

authors argued that the difference is due to the lipidic composition of the cell-membrane: the sterol present in mammalian cells is cholesterol, whereas plant cells have sitosterols, stigmasterols and campesterols. Also, the respective glycerolipid fatty acid chains are different, with stearic and arachidonic acids in mammalian cells, and linoleic and linolenic acids in plant cells.

Endocytosis has different regulated gates of entry into the cell, which can initially be divided in two main categories: phagocytosis (cell eating) or pinocytosis (cell drinking) [59,60]. Phagocytosis takes place only in specialised mammalian cells, however, pinocytosis occurs in all cells by four different mechanisms: macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis or lipid-rafts (i.e., clathrin- and caveolin-independent endocytosis). The kind of endocytosis may depend not only on the CPP but also on the type of cargo attached to it.

For Tat peptide, two different mechanisms have recently been described: caveolin-mediated endocytosis and macropynocitosis [61,62]. A GST-Tat-EGFP construct (using only the CPP region of Tat) was internalised through caveolae as proven by slow kinetics of internalisation, endosome-resistant treatment to Triton X-100, colocalisation with caveolin-1 and cholera toxin, and an internalisation sensitive to drugs that reduce lipid raft formation (cyclodextrin or cytochalasin D) [47,61]. Internalisation via caveolae is a favourable pathway for drug delivery as it is non-acidic and nondigestive.

On the other hand, the fusogenic Tat(CPP)-Cre protein was found to colocalise with the endocytic marker FM4-64, to be sensitive to cholesterol depletion and to not colocalise with caveolin-1, thus imputing macropinocytosis as the internalisation mechanism followed [62].

The fact that R_8 internalised less when incubated with the macropinocytosis inhibitor 5-(*N*-ethyl-*N*-isopropyl)amiloride proved that at least a fraction of the peptide is internalised via said mechanism [55]. Similar experiments were performed for penetratin, showing that macropinocytosis plays a minor role in its uptake.

For hCT(9–32)-br and SAP CPPs it has been shown that the internalisation takes place via lipid-rafts, as clear colocalisation with cholera toxin was observed and the uptake was decreased in the presence of the cholesterol-sequestering agent methyl-β-cyclodextrin [24].

It is also important to note that enantio versions of CPPs have been shown to translocate the cell-membrane, thus pointing to a receptor-independent mechanism [1,63,64].

2.3. Third step: CPP intracellular pathway

Cellular internalisation mechanisms determine the cell-compartment destination. Clathrin-mediated endocytosis implies that the degradative route is followed, from early endosomes to late endosomes, and ultimately to lysosomes (Fig. 1(3a)). On the other hand, if the caveolin-mediated route is taken then the vesicles are targeted either to the Golgi apparatus or to the endoplasmic reticulum (ER) (Fig. 1(3b)) [65]. Moreover, when the vesicles are first directed to the Golgi, a retrograde transport to the ER exists.

Fischer et al. [49] have suggested that cationic CPPs enter the cytoplasm through a retrograde transport. Working with brefeldin A and with nordihydroguaiaretic acid (NDGA), which interfere with the integrity of the Golgi and the trans-Golgi network, they found that the cellular uptake of R_9 and Antp was diminished considerably. In the case of Tat, its fluorescence was reduced by brefeldin A but increased with NDGA. Moreover, they observed partial colocalisation with the cell-permeable Golgi-tracer Bodipy ceramide for Tat and Antp peptides. These CPPs did not traffic into lysosomes as confirmed by an absence or near-absence of colocalisation with Lysotracker, an acidic compartment-specific probe.

2.4. Intracellular drug release: endosomal escape

As the principal internalisation mechanism is endocytosis, it seems that most of the cell-penetrating peptides are not able to directly translocate through membranes; they somehow interact with, or insert into, the cell-membrane, thereby triggering the endocytic machinery. As CPPs eventually become trapped in the endosome, an escape route to the cytoplasm must be exploited before the onset of lysosomal activity so that the cargo can be released in the cytosol to exert its biological effects (Fig. 1(3c)).

Different approaches to endosomal escape have been suggested, from acid labile bonds to endosome-disrupting peptides or laser triggered endosome aperture.

It is known that there is a decrease in vesicular pH during endosomal trafficking, from approximately 7.4 to 5.0, suggesting the use of acid-sensitive linkers to release the cargo covalently bound to the peptide. This strategy was first described for a model peptide, neuropeptide Y (NPY), which has receptors expressed in neuroblastomas [66]. Upon comparison of two daunorubicin-NPY conjugates having either a stable amide bond or an acid-sensitive hydrazone linker, only the former was found to be active. Afterwards, this strategy was successfully applied in the case of hCT(9–32) also linked to daunorubicin to achieve similar results [67].

Another proposed strategy is the use of endosome-disrupting peptides. The 20 amino acid N-terminal region of the influenza virus hemagglutinin protein, HA2, is used for this purpose, as it is able to destabilise lipid membranes at low pH [62]. It was used to asses the Tat-Cre mediated recombination, whereby an increase in recombination was found when Tat-HA2 was added to Tat-Cre, suggesting that greater release into the cytoplasm had occurred [62]. Another endosome-disrupting peptide is 4_3E peptide (LAEL–LAEL–LAEL), which disrupts endosomes via a conformational change from a random coil at pH 7.4 to an α-helix at pH 5.0 [68]. Adding 4_3E peptide to a mixture of 4_6 (a cationic amphiphilic CPP) and plasmid DNA encoding firefly luciferase led to a remarkable increase in transfection level.

Endosomal release of a cargo peptide fused to a fluorescently labelled CPP by laser illumination has recently been reported [69]. The release is thought to occur as a result of damage caused by short-lived oxygen species generated by the applied

light. The authors propose the use of fluorophore-labelled CPP as a phototrigger to induce endosomal escape. The photochemical endosomal opening is a relatively non-cytotoxic treatment, as proved by the observed biological response after laser irradiation and the retained cell morphology 24h after treatment.

Currently, the majority of in vitro experiments for disruption of endosomal membranes imply the use of chloroquine or sucrose. These compounds neutralise the endosomal pH, thereby blocking the transfer of peptide to late endosomes. However, this method is not feasible from a therapeutic point of view since the compounds lack membrane specificity, and problems in the co-administration could arise.

Once the endosomal release is achieved, another problem may emerge if the cargo is covalently bond to the CPP. If the cargo linked to the CPP is unable to carry out its biological activity, then the need to cleave the cargo from the CPP arises. This problem can be solved if the peptide and cargo are connected via a disulphide bond: upon arrival in the cytoplasm, the CPP-cargo bond rapidly is cleaved by the action of cytoplasmic glutathione.

3. Common features among CPPs and relevance to the different internalisation steps

3.1. Positively charged amino acids

A feature shared among CPPs is a high degree of positive charges, due to their content of the basic amino acids lysine and arginine. It has been postulated that this characteristic is important for the initial step of internalisation, the previously explained interaction of cell-surface proteoglycans with sulphate groups. Positively charged amino acids may also be important for the subsequent interaction of the CPP with the nearby phosphate heads of the membrane lipids. At physiological pH, both lysine (pK_a, 10.5) and arginine (pK_a, 12) are protonated, and hence interact with negatively charged sulphate and phosphate groups of the extracellular cell matrix. However, it must be taken into account that due to the pK_a difference between lysine and arginine, K-rich peptides suffer partial deprotonation, whereas R-rich peptides have to scavenge anions in order to minimise charge repulsion [70–72].

A study comparing different homopolymers of arginine and lysine established that those with arginine were highly internalised [73], therefore, the guanidinium headgroup was believed to be the crucial structural component for the internalisation. This idea was further supported by the fact that homopolymers of citrulline, which has a urea instead of a guanidinium moiety, are not internalised. Thus, it was concluded that the special ability of the guanidinium group to form bidentate hydrogen bonds with phosphate or sulphate groups was the key (Fig. 2). The number of arginines required for optimal cell-penetration in polyarginines seems to be between 7 and 15, depending on the techniques and the cell line used [56].

The crucial role of the guanidinium head group in translocation has been assessed, since modifications in the amino acid side chain, peptide backbone or linearity of the oligoarginines do not seriously affect cellular internalisation.

To establish the importance of the backbone, a series of polyguanidine peptoid derivatives were synthesised, preserving the 1,4-backbone spacing of arginine side chains but having an oligoglycine backbone without stereogenic centers. These molecules were taken up only slightly less than the corresponding D-arginine peptides, suggesting that the hydrogen bonding properties of the peptide backbone do not seem to be important for cellular uptake [74]. Also, peptoid derivatives with different numbers of methylene groups in the side chain were synthesised. Those having longer side chains were taken up to a greater extent. To distinguish between the possible contributions of the hydrophobicity of the methylenes or the flexibility of the side chain on the internalisation rate, peptoid derivatives containing cyclohexyl side chains were prepared. As the uptake was lower when the degrees of freedom decreased, the conformational flexibility of the linear alkyl chain appeared to be important for cellular uptake. Further evidence that the backbone is not essential for translocation is that a series of guanidine-rich oligocarbamates were able to rapidly cross the cell membrane, even faster than the polyarginines of the same length [75]. Therefore, the backbone is only a scaffold which serves to expose the guanidinium groups.

The linear structure of the arginine-rich peptides is also of no importance to translocation, as a series of branched-chain arginine-rich peptides have been shown to translocate the cell membrane very efficiently [6,76].

Nevertheless, it is surprising that a highly cationic molecule can so efficiently cross the cell membrane. Some light on this matter has emerged from a study by Rothbard et al. [77]. Once the positive charges are neutralised from the interaction with anionic cell-surface groups, the less polar ion pair complex can cross the cell membrane. To support this hypothesis, the octanol/water partitioning of octaarginine in the absence or presence of a fatty acid salt (sodium laureate) was studied. Whereas the peptide alone stayed at the water layer, it moved into the octanol layer upon addition of the fatty acid salt (>95%). Surprisingly, ornithine oligomers in the presence of the fatty acid salt stayed in the aqueous layer, again underlying the importance of the guanidinium moiety and its capacity to form effective bidentate hydrogen bonds. This was further supported by checking the uptake capacity of mono- or dimethylated arginine oligomers, in which the uptake was reduced by 80% and more than 95%, respectively.

Apart from polyarginines, most CPPs contain arginine, including Tat, penetratin, 4_6, pVec, SynB peptides and SAP.

In the case of SAP, three different positively charged amino acids were chosen to modify the hydrophilic face of the amphipathic PP II helix: His, Lys and Arg. Peptides bearing Arg proved to be the most efficiently internalised by plate fluorimetry and CLSM [78].

Individual substitutions of the Tat(49–57) cationic residues by alanine caused a 70–90% decrease in cellular uptake [74]. Furthermore, Vives et al. working with variants of Tat(48–60) in which the positively charged amino acids were deleted or substituted, observed that translocation activity was indirectly proportional to charge level [79].

Although the majority of authors claim that the guanidinium group of arginine is more effective for cell-uptake than other positively charged groups there is one report against this idea. Experiments have been performed in which large macromolecules with different lengths of arginine or lysine homopolymers were delivered to various cell lines. The biotinylated peptides were complexed with streptavidine and the 8 or 10 residue long polylysines proved to be the most effective in the transduction of the complex [15].

Also, a series of publications have made use of oligolysine tails to promote the uptake of various oligonucleotide analogues [80,81].

It is worth noting that, apart from being an essential feature for membrane translocation, positive charges are very useful for non-covalently binding negatively charged cargo (e.g., DNA or RNA).

Even oligoguanidinium vectors, non-peptidic molecules that bear the guanidinium moiety, have been described as very efficient carriers [28]. Nonhydrolysable tetraguanidinium compounds, formed by highly preorganised chiral bicyclic guanidinium subunits linked through thioether spacers, underwent more efficient cell-membrane translocation than Antp or Tat CPPs. Interestingly, these cell-penetrating vectors, bearing an array of highly basic guanidine groups, are able to reach mitochondria.

3.2. Hydrophobicity

Hydrophobicity is another common feature shared among the majority of CPPs. It was initially thought that this characteristic was essential for the interaction with the lipidic part of the cell-membrane, but its function became unclear upon discovery of the role of HSPGs in the first internalisation step.

The cellular uptake of penetratin has been described to be dependent on the presence of a central hydrophobic core (W6, F7). Substitution of the two tryptophan residues of penetratin (positions 48 and 56) with phenylalanines did not lead to significant differences in cellular uptake in unfixed cells [48], whereas in fixed cells, it was observed that this substitution prevented translocation of the peptide [1,82].

For Tat peptide, the attachment of a small hydrophobic molecule, biotin, to the peptide caused a 6-fold increase in cellular uptake [83]. In addition, insertions of aminocaproic acid groups in the peptide backbone led to a stronger cell-association [84].

A family of carrier peptides based on the hydrophobic core (H region) of a signal peptide has been described to be efficiently internalised in HEL cells [85–88]. The peptides included two different sequences: one derived from Kaposi fibroblast growth factor (AAVALLPAVLLALLAP) and the other from integrin β_3 (VTVLALGALAGVGVG).

Upon varying the content of hydrophobic Leu and hydrophilic Lys amino acids in Hel amphipathic peptides, it was found that the peptide with the highest proportion of hydrophobic amino acids, Hel 13-5 (KLLKLLLKLWLKLLKLLL), was the most efficient at delivering DNA [89]. It was also determined that the hydrophobic region is important for the formation of aggregates when delivering non-covalently DNA. It was then hypothesised that the aggregate state may be important both for cellular-uptake and for preventing DNA degradation. Moreover, the hydrophobic amino acids were needed for stabilising the α-helical structure.

Another example of the importance of hydrophobicity is the lipophilisation at the N-terminus of mastoparan (MS, INLKALAALAKKIL) or α-helix model peptide (HM, LARLLARLLARL) [90]. In the case of MS, it was modified with a single acyl chain, a dialkylcarbamoyl group or a cholesteryloxycarbonyl group, whereas HM was only modified with an acyl group having a single chain. These modifications stabilised the α-helical structure and the self-assembly with a defined number of monomers. Gene transfer efficiency results revealed that in the presence of chloroquine, some MS and HM lipophilic derivatives were able to deliver DNA plasmids even more efficiently than Lipofectin, reaching or even surpassing its levels of internalisation.

Polyarginines were also modified with hydrophobic moieties rending an improved CPP version [91]. Octaarginine was lipophilised at the N-terminus with a stearyl, lauryl or cholesteryl group, and it was observed that the derivative bearing the stearyl group had the best transfection efficiency properties, improving the octaarginine internalisation level by 2 orders of magnitude, and reaching the levels of Lipofectamine. This effect was also observed for Tat(48–60), as the stearylated derivative was 2 orders of magnitude higher in gene transfection. Using dynamic light scattering it was determined that the complexes of stearyl-octaarginine with plasmid DNA form large aggregates compared with octaarginine or lipofectamine alone. In this case the authors argue that the hydrophobic moiety is mainly positioned on the outside of the aggregate, contributing to the absorption of the complex into the cell-membrane. It is also explained that the complexes with the stearyl moiety are stabilised, by both hydrophobic and ionic interactions, producing a positively charged complex that could better interact with cell-surface [92]. Furthermore, chloroquine treatment generated similar results, hence the authors believe that the octaarginines conjugated with hydrophobic moieties are able to disrupt endosomes.

The uptake of SAP was improved when it was coupled to caproyl or myristyl fatty acyl groups, especially in the case of the latter, which has the longer chain [93]. Interestingly, only this SAP derivative was found to interact with neutral DOPC monolayers.

As there are CPPs composed exclusively of polar residues, such as polyarginines and polylysines, it would seem that hydrophobic moieties are not essential for translocation. However, recent evidence argues against this notion. The importance of hydrophobicity, in the presence of a fluorophore, was recently highlighted in polyarginine uptake experiments [94]. Normally, the comparison of the cellular uptake between a peptide with or without a fluorophore cannot be made because a fluorescent label is required to quantify peptide internalisation, but in this study, the authors used ^{19}F-NMR instead of the common fluorescent techniques. The fluorescently-labelled peptide had a greater affinity for the cell membrane as compared to that of the non-labelled peptide. The authors argue that the fluorescent label acts as an anchor to the cell

membrane, immobilising the polyarginine onto the cell surface and providing the peptide entry into the cell.

For all of the aforementioned reasons, it is believed that in addition to electrostatic and hydrogen bonding interactions, hydrophobic interactions also play a key role in CPP uptake.

In the case of non-peptidic cell-penetrating vectors, the importance of hydrophobicity has also been reported. A tetraguanidinium compound with a *tert*-butyldiphenylsilane group (TBDPS) placed at one terminus of the tetraguanidinium chain was 4-fold more efficient in translocation than the same vector without this moiety [28].

3.3. Amphipathicity

A consequence of having both hydrophilic and hydrophobic amino acids is that many CPPs are amphipathic.

In the context of peptides, the amphipathicity may result from the primary or secondary structure [95]. Peptides with primary amphipathicity are assembled from a hydrophilic and a hydrophobic region which are normally divided by a spacer domain. On the other hand, secondary amphipathicity is achieved when the peptide conformation is such that all of the polar residues point to one side and the non-polar ones to the opposite side.

Two main groups of primary amphipathic peptides have been described: signal peptides (SP) and fusion proteins (FP). As a hydrophobic sequence, the SP family contains derivatives of the SP Ig(v) 40 light chain of *Caiman crocodylus* (MGLGLHLLVLAAALQGA), whereas the FP family contains analogues of the gp41 fusion protein of HIV1 (GALFLGFLGAAGSTMGA). As a hydrophilic domain, both families contain the nuclear localisation sequence of the large T-antigen of SV40 (PKKKRKV). The SP family has proven to be effective in the delivery of covalently linked porphyrins [96] and oligonucleotides [97,98] and also of complexed RNA or DNA [98]. DNAs encoding β-galactosidase or luciferase and antisense DNA have been internalised with CPPs from the FP class [99]. Another efficient CPP having primary amphipathicity is Pep-1 [100–102], for which this characteristic has been postulated to be responsible for its strong interaction with lipidic membranes [103].

CPP amphipathicity as a result of secondary structure has been reported in many examples, which can be divided into two main groups: secondary amphipathicity due to α-helical conformation or secondary amphipathicity from PP II structure. Tat(47–57) and Antp(43–50), peptides derived from the membrane destabilising peptide JST-1 and MAPs are examples of the first class. The modelled structure of Tat(47–57) was shown to be an amphipathic helix, with hydrophobic residues pointing to one side of the helix and the hydrophilic ones pointing to the other face [104]. Moreover, when three positions of the peptide were substituted by α-helical promoting Ala, a 5-fold internalisation increase was reached. Strikingly, when the content of Ala residues was expanded to six and the Arg limited to three, the improvement in uptake was 33-fold.

The region 43–50 of Antp(43–58) is strongly folded in an α-helical structure, but there is also a minor conformer with the N-t and the C-t regions in an extended conformation [105]. Surprisingly, this extended conformation permits the formation of aggregates. The content of α-helix conformation increases as the environment approaches that of a cell-membrane (water, perfluoro-*tert*-butanol and SDS). An analogue with Pro in position 50 [Pro50]pAntp also adopts the α-helical structure and is internalised in cells, but in contrast to the parent CPP sequence, does not show conformation dependence on the media [40]. A cyclic version of Antp did not exhibit cellular uptake, denoting the importance of peptide conformation for internalisaton and as well as for amphipathicity.

Other peptides with secondary amphipathicity due to an α-helix conformation are ppTG1 (GLFKALLKLLKSLWKLLLKA) and ppTG20 (GLFRALLRLLRSLWRLLLRA), derived from the membrane destabilising acidic peptide JST-1, in which the glutamic acid residues are substituted by Lys or Arg, respectively. These CPPs are able to adopt an α-helical conformation, show membrane destabilising properties (as assessed by the liposome leakage assays) and also DNA-binding and gene transfer abilities [106]. It is worth noting that the in vitro gene transfer ability of ppTG1 is in the same range as other commercial available transfection reagents, such as the cationic lipid Lipofectin or the dendrimer Superfect. The authors claim that there is a correlation between peptide conformation, liposome leakage and gene transfer efficiency, as a derivative with the leucines replaced by valines, ppTG30, adopted a β-sheet structure and was not able to induce liposome leakage or to efficiently deliver plasmid DNA.

To study the influence of amphipathicity in cellular uptake, a series of derivatives of model amphipathic peptide (MAP) were developed [107]. Detecting uptake by HPLC, it was initially established that amphipathicity was crucial for internalisation [108,109] but afterwards, using an online protocol for confocal laser scanning microscopy including a washing step, it was shown that amphipathicity is not essential, as both amphipathic and nonamphipathic peptides crossed cell-membranes [110].

Another report against the idea that amphipathicity is a key feature for cell-membrane translocation is the case of the analogue of pAntp, AP-2AL (RQIKIWFQAARMLWKK) [111]. This derivative is more ordered in an α-helical conformation, resulting in a more amphipathic peptide which in turn leads to an increased lipid binding affinity. In contrast to pAntp, AP-2AL is capable of provoking the leakage of calcein from vesicles. Whereas AP-2AL translocates the phospholipid bilayer and destabilises the inner part of it, pAntp stays on the outer leaflet and it is not able to translocate. It has therefore been hypothesised that the AP-2AL derivative, in contrast to pAntp, crosses the cell-membrane by pore formation, and also that the helical amphipathicity of AP-2AL does not correlate with the characteristics of pAntp that cause its cellular uptake.

CPPs having secondary amphipathicity due to polyproline II structure include SAP, Tat(48–61) and agents based on a polyproline helix scaffold. In the case of SAP, a 50% proline-content leads to a PP II structure of 3.0 residues per turn [78]. A secondary amphipathic peptide can then be built by placing hydrophobic residues on one side of the helix and hydrophilic amino acids on the opposite face. Recently, it has been reported that Tat(48–61) adopts a polyproline II (PPII) secondary

structure, leading to secondary amphipathicity, as the nonpolar residues are mainly positioned at one face of the helix and the charged ones are pointing to the other side [112].

Finally, a series of agents based on a polyproline helix scaffold have been proposed as efficient cell-penetrating vectors [113]. Hydroxyproline monomers were functionalised by O-alkylation with hydrophobic or cationic moieties. Afterwards, amphipathic agents were synthesised taking into account the 3.0 residues per turn periodicity of the PP II helix, with one face of the helix containing hydrophobic moieties and the other two with cationic groups. After verifying by CD that these molecules can adopt a PP II structure, they were tested as intracellular delivery agents, proving to be highly efficient.

4. Relevance of CPP self-assembly

The majority of CPPs contain positively charged groups, mainly guanidinium moieties, but also amino groups, as well as hydrophobic groups. It has also been observed that the location of these groups in the sequence is not trivial; the optimal positioning being that which favours maximum amphipathicity (primary or secondary), or a maximum level of separation between polar and non-polar parts. It is also well known that amphiphilic compounds have a high tendency to aggregate when placed in water or hydrophilic solvents.

It thus follows that certain CPPs can self-assemble, suggesting that CPPs can be internalised as monomers or aggregates. Currently, no clear relationship between aggregation and internalisation has been established, and evidence of aggregation has only been provided for some CPPs (Fig. 3).

The first article reporting that the third helix Antennapedia Homeodomain translocates biological membranes [1] includes an explanation of how the different peptides derived from its sequence form aggregates in the presence of SDS. The authors maintain that the peptides will behave in the same manner in a lipid bilayer than in a cell membrane environment. Interestingly, following internalisation into E15 rat embryos, the peptides were recovered as multimeric complexes.

Working with a solution of biotinylated transportan and using polyacrylamide electrophoresis, it was found that the transportan molecules are assembled into different multimers, wherein the dimeric form predominates. Taking into account these results, the authors suggest that at concentrations of 1 μM and higher, CPP binding to the cell membrane occurs in the form of oligomers or micelles.

The importance of aggregate formation for efficient gene-transfer has been described [114]. Peptides such as $4_6\Delta8$ (LARLLARLLARLLARL) or Hel 11-7 (KLLKLLLKLWKKLLKLLK) are able to form aggregates with DNA and also have the ability to transfer DNA. In the case of $4_6\Delta12$, which is not able to form aggregates, the transfection efficiency is poor compared to that of peptides that form aggregates. Moreover, aggregate formation prevents DNA degradation.

As for hCT(9–32), self-assembly of the natural human calcitonin hormone has been described [115]. Fibril formation was assessed by turbidimetry measurements and electron microscopy, whereby large, helically organised fibrils of 8–17 nm in diameter were found.

Other CPPs, like Pep-1 or MAPs have also been reported to form aggregates in solution [103].

4.1. Sweet arrow peptide

In 2004 our laboratory described a novel group of CPPs: amphipathic Pro-rich peptides [78]. The principal advantages of these compounds are non-cytotoxicity, non-viral origin and high solubility in aqueous media. The discovery stemmed from combining findings from two different research projects. Firstly, it was known that the N-terminal Pro-rich repetitive domain of γ-zein, $(VHLPPP)_8$, can interact with PC (phospatidylcholine) liposomes and form deposits on the liposome membrane [116]. Secondly, it had also been shown in our lab that a linear polyproline peptide (Pro_{14}) labelled with fluorescein at the N-terminus could cross the membrane of NRK cells [117]. The design was based on a minimum proline content of 50% in the sequence, in order to conserve the 3.0 residues per turn

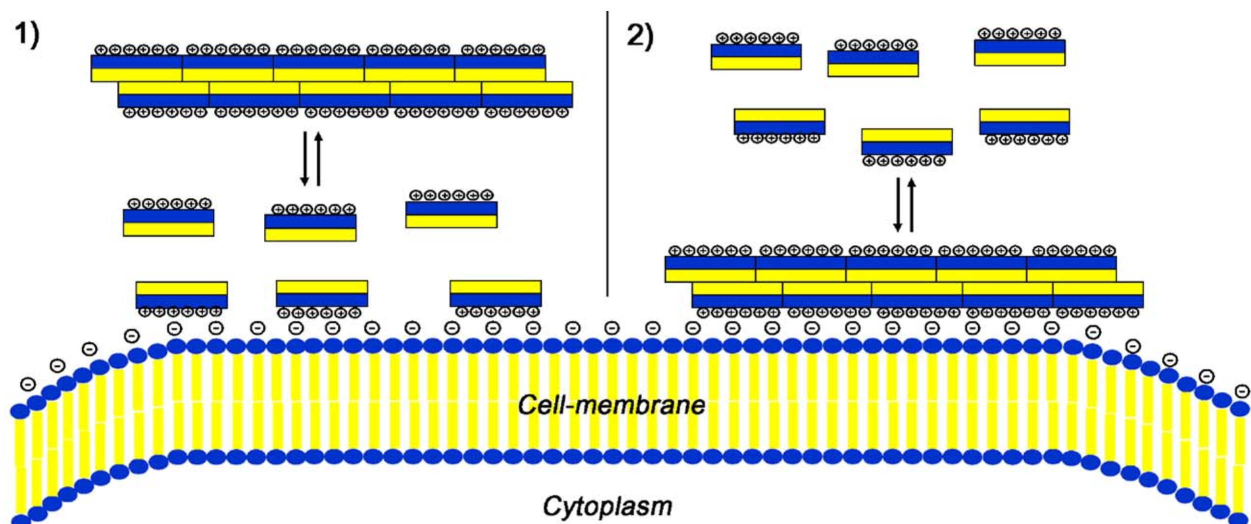

Fig. 3. Alternative models for the internalisation of self-assembling CPPs where either the CPP monomer (1) or the CPP aggregate (2) are internalised.

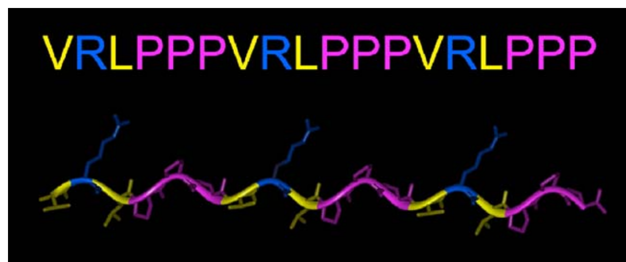

Fig. 4. SAP formula and structure, showing amphipathic secondary structure.

periodicity of polyproline II (PP II) structure. Hence, an amphipathic helix could be formed by placing hydrophobic residues at positions $i/i+2$, $i+6/i+8$, $i+12/i+14$…, hydrophilic residues at $i+1$, $i+7$, $i+13$…, and prolines at the remaining positions. In an analogy to the N-t domain of γ-zein, the hydrophobic residues chosen were valine and leucine, yielding the sequence $(VXLPPP)_n$ (Fig 4). The positively charged H, K or R were selected as hydrophilic residues, and monomers, dimers or trimers were synthesised ($n=1$–3). Measuring internalisation in HeLa cells, the best candidate found was the trimer with arginine residues, $(VRLPPP)_3$. Due to its non-cytotoxic character and its permeability through the cell membrane, the CPP was named Sweet Arrow Peptide (SAP).

An interesting property of the γ-zein octamer sequence is its strong tendency to aggregate, as shown by the CD spectra dependence on concentration as well as by the fibrils observed by AFM and TEM [118,119]. The fibrils are 20 (±2) nm wide up to 1000 nm long. The inner part of the fibrils has cylindrical filaments 3 (±0.7) nm wide. The proposed aggregation model is the formation of cylindrical micelles due to hydrophobic and hydrophilic interactions. The growth of the fibril seems to be longitudinal (i.e., the molecules are aligned in the direction of the fibril axis) because fibril width has been shown to be independent of molecule length. It is believed that the first micelles formed have a diameter of 3 nm, and a secondary aggregation of several cylindrical micelles follows, leading to the formation of 20 nm fibrils. The first micelles are formed because of hydrophobic interactions, by which non-polar residues are positioned inside and polar residues are positioned outside. The latter occurs during the second aggregation step to form cylindrical micelles and requires counterions because ionic interactions are taking place.

Thus, we set out to determine if the self-assembling pattern was also present for SAP CPP. It was found that the CD spectrum of $(VRLPPP)_3$ is typical of a PP II secondary structure, with an intense negative band at 203 nm. However, at 25 °C it is not possible to see the weak positive band at 228 nm. Spectra at different concentrations exhibit a self-assembly pattern typical of amphipathic peptides. Flexible peptides able to adopt amphipathic secondary structures are in equilibrium between disordered non-amphipathic and ordered amphipathic structures. An increase in peptide concentration causes an increase in aggregation, which in turn shifts the equilibrium towards the ordered structure. This displacement can be monitored using CD. In the case of $(VRLPPP)_3$, increasing the concentration from 5 to 50 μM enhances molar ellipticity, most likely indicating that aggregation is occurring. From 50 to 100 μM, molar ellipticity remains constant, denoting that an equilibrium state has been reached, wherein fibrils have already been formed (Fig. 5). To visualise the aggregates suggested by CD, drops of a 50 μM SAP aqueous solution was freeze-fixed, freeze-dried and covered with Pt over a

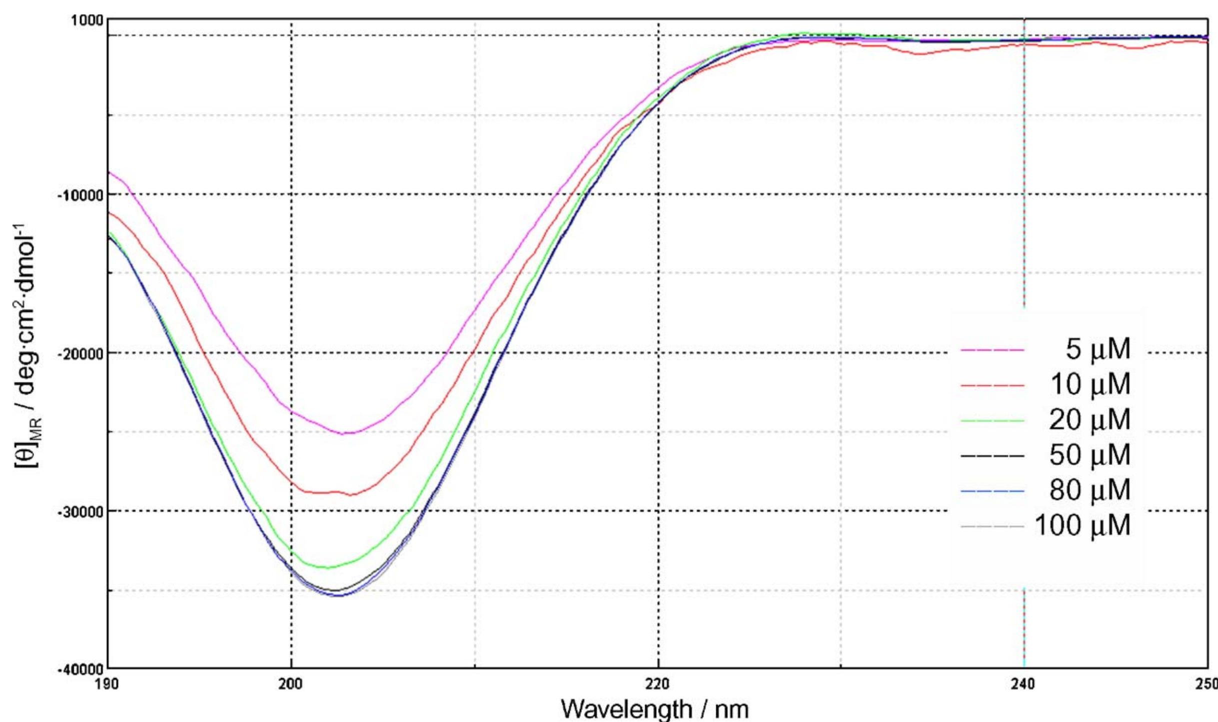

Fig. 5. CD spectra of SAP at varying concentrations ($c=5$–100 μM) in 10 mM aq. phosphate buffer at pH 7.

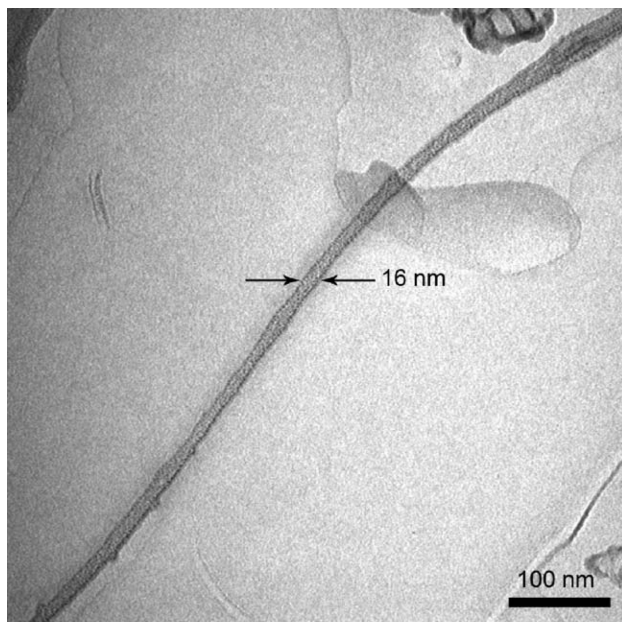

Fig. 6. Transmission electron microscopy image of the replica obtained after freeze-fixation and freeze-drying of a 50 μM aqueous solution of SAP over an uncoated coverslip.

coverslip for observation by TEM. This process allows preservation of any supermolecular structure present in the peptide solution. A concentration of 50 μM was chosen for two reasons: it had been observed that the final aggregated state is reached at this concentration, and also because it is the concentration used in cell internalisation assays. TEM micrographs of the replicas show fibrils of 16 (±3) nm width and variable length (Fig. 6). Thus, we believe that the same aggregation model of the octamer can be applied in the case of SAP peptide (Fig. 7).

As with other self-assembling CPPs, the internalised species (i.e., monomeric or oligomeric) have yet to be identified.

5. Experimental part

5.1. Materials

Fmoc-Nα-protected amino acids were obtained from IRIS Biotech GmbH (Germany). The 2-chlorotrityl chloride resin was purchased from CBL-PATRAS (Greece). Coupling reagents: Benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate, PyBOP, was purchased from Novabiochem (Switzerland); 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) was purchased from Albatros Chem Inc. (Canada). Trifluoroacetic acid (TFA) was purchased from Scharlab S.L.(Barcelona). Piperidine, dimethylformamide (DMF), dichloromethane (DCM) and acetonitrile were purchased from SDS (France). *N,N*-diisopropylethylamine (DIEA) was obtained from Merck (Germany). Triisopropylsilane (TIS) was obtained from Fluka (Switzerland).

5.2. Synthesis and chromatography

SAP was synthesised by solid phase synthesis using the 9-fluorenylmethoxycarbonyl/*tert*-butyl (Fmoc/*t*Bu) strategy. 2-Chlorotrityl resin, Nα-Fmoc-protected amino acids (2 eq.)/TBTU (2 eq.) and DIEA (6 eq.) were used. As protecting group for the side chain of Arg 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl (Pbf) was used. The Fmoc protecting group was cleaved by treatment with a solution of 20% piperidine in DMF (2×10 min). For the incorporation on the growing peptide-resin of the Fmoc-Arg(Pbf)-OH, the TBTU coupling reagent was replaced by the more potent phosphonium salt PyBOP (2 eq.) and it was pre-activated for 10 min prior to the addition of the amino acid to the peptide-resin.

SAP was cleaved from the resin by treatment with 95% TFA, 2.5% TIS, 2.5% water for 1h and identified at $\lambda = 443$ nm by analytical RP-HPLC [Waters 996 photodiode array detector equipped with the Waters 2695 separation module, the

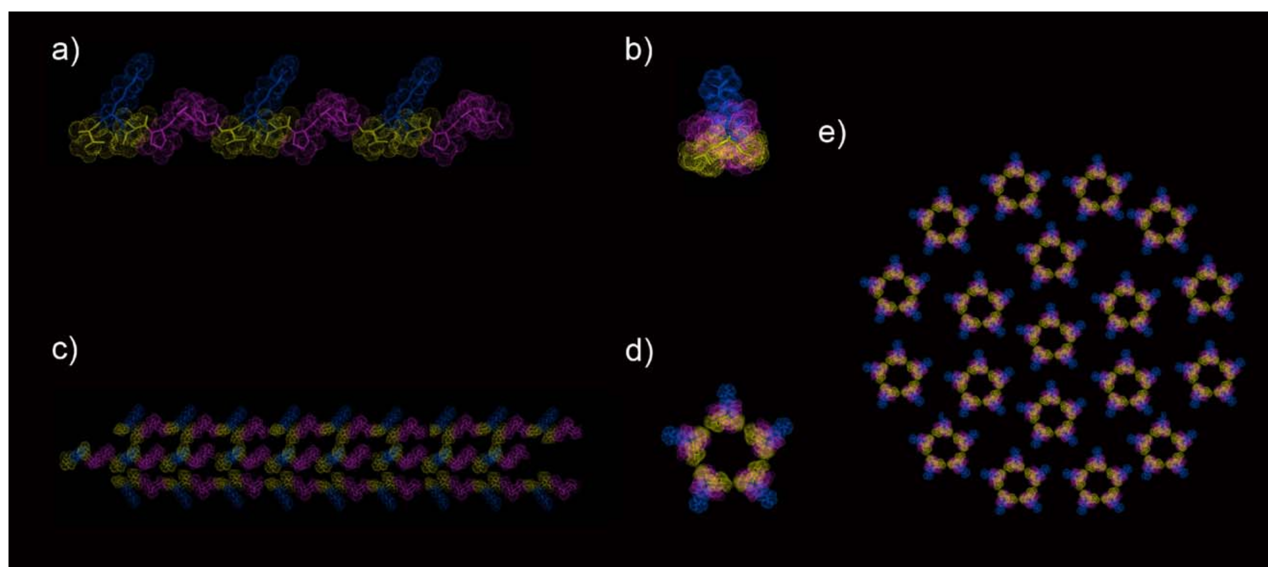

Fig. 7. Proposed model for the SAP self-assembly (i.e., formation of cylindrical micelles).

Symmetry column (C18, 5 μm, 4.6×150 mm) and the Millenium software; Flow=1 ml/min; Gradient=5–100%B in 15 min (A=0.045% TFA in H_2O, B=0.036% TFA in acetonitrile)]. The purification was done in a semipreparative RP-HPLC [Waters 2487 Dual λ Absorbance Detector equipped with a Waters 2700 Sample Manager, a Waters 600 Controller, a Waters Fraction Collector, a Symmetry® column (C18, 5 μm, 30×100 mm) and a Millenium chromatography manager software; Flow=10 ml/min. Gradient=5–20%D in 5 min; 20–70%D in 30 min; 70–100%D in 5 min (D=0.1% TFA in acetonitrile)]. SAP was characterised by MALDI-TOF mass spectrometry (Vogayer-DE RP MALDI-TOF, PE Biosystems with a N_2 laser of 337 nm).

5.3. Circular dichroism

Circular dichroism spectra were recorded with a Jasco 810 UV-Vis spectropolarimeter, a Peltier CDF 426S/426L and a temperature control JULABO F25 programm. The spectra were obtained in a wavelength range of 190–250 nm at a spectral bandwidth of 1 nm, with a time response of 4 s, a scan speed of 10 nm/min and a step resolution of 0.1 nm. Each spectrum was the average of three accumulations. Spectra were measured at concentrations ranging from 5 μM to 100 μM of peptide solved in 10 mM phosphate buffer at pH and were recorded at 25 °C. The blank was subtracted from each peptide spectrum. Molar ellipticity is expressed per decimal residue.

5.4. Transmission electron microscopy (TEM)

Drops of aqueous samples of the peptides at 50 μM concentration were deposited over uncoated cover slips. Cover slips were freeze-fixed by projection against a copper block cooled by liquid nitrogen (−196 °C) using a Cryoblock (Reichert-Jung, Leica, Germany). The frozen samples were stored at −196 °C in liquid nitrogen until subsequent use. Samples were freeze-dried at −90 °C and coated with platinum and carbon using a freeze-etching unit (model BAF-060, BAL-TEC, Liechstenstein). A rotatory shadowing of the exposed surface was made evaporating 1 nm platinum-carbon at an angle of 6° above the horizontal, followed by 10 nm of carbon evaporated at a 90° angle. The replica was separated from the cover slip by immersion in concentrated hydrofluoric acid, washed twice in distilled water and digested with 5% (v/v) sodium hypochlorite for 5–10 min. The replicas were washed several times in distilled water and collected on Formvar-coated copper grids for electron microscopy. All electron micrographs were obtained using a Jeol JEM 800 MT electron microscope (Japan) operating at 80 KV. Images were obtained on a CCD camera Megaview III (ISIS), Münster, Germany. Three samples were prepared following the sample procedure and results obtained by TEM imaging were reproducible.

6. Concluding remarks

In summary, we have seen that CPPs share properties and how these properties may be related to specific steps of the various mechanisms of cellular internalisation. Thus, positive charges are first used to interact with sulphates, carboxylates and phosphates of the extracellular matrix. Hydrophobicity enables a CPP to bind more tightly to a cell membrane, or to penetrate a bilayer and achieve endosomal release. Moreover, cationic and hydrophobic residues in CPPs are positioned for maximal separation, often leading to amphipathicity. Lastly, many CPPs are known to self-assemble, however, the role of this property in cellular uptake remains to be elucidated.

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